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Usefulness of Cholinesterase Measurements

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16. ABSTRACT

We think that Hill's (1988) efforts to collate and publish his data base of "normal" brain ChE values for so many avian species is commendable but the data must be used with care. A standardized method outlining techniques for collection and storage as well as assay parameters such as cuvette type and homogenization procedures is essential before we can utilize data bases generated by Hill and others to their full extent. The technique should be as rigorous and biologically realistic as possible yet still capable of being conducted in a field laboratory by personnel with minimal training in biochemical techniques. Most importantly, proper quality control and assurance procedures should be adhered to by all laboratories conducting the tests, regardless of whether their purpose is field diagnostics or rigorous research. Until such time as interlaboratory data can be more comparable, we will need to continue to rely on our own data sets, the 2-PAM reactivation technique, or comparisons of exposed birds to unexposed or "control" animals collected concurrently and use published control values only as a check to determine if we find similar trends among species.

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LETTER TO THE EDITOR . . .

The Usefulness of Cholinesterase Measurements

One of the assays most frequently used by toxicologists for diagnosing exposure of wildlife to organophosphate or carbamate compounds is measurement of the inhibition of cholinesterase (ChE) activity in brain tissue. Major problems in the use of this assay are determining what constitutes a "normal" ChE value for a given species and how much an individual's value must be depressed before it is indicative of exposure to or death from a ChE-inhibitor. Hill's (1988) paper on "Brain cholinesterase activity of apparently normal wild birds" recently published in the *Journal of Wildlife Diseases* contains the first compilation of normal values of bird brain ChE activity generated by a single laboratory over many years. A data base (Hill, 1988) such as this has applicability when assessing effects of ChE-inhibitors on avian species and is likely to be used in regulatory decisions setting guidelines for acceptable amounts of ChE depression caused by proposed pesticide use. The publication of this data base has stimulated us to respond with this discussion on the current use, and the potential for misuse, of ChE measurements from wild animals.

Hill's (1988) data begins to fill the void of information about normal ChE values of wild animals. It will enhance the ability to document field poisoning that may have gone undiagnosed because concurrent control animals could not be collected for the myriad of reasons often confronting field biologists (e.g., widespread application of pesticides, rarity of the species involved, or a concern for sacrificing apparently healthy animals). However, those who may wish to utilize the data set in this manner should pay particular attention to Hill's warning that the values he presents are reproducible only if his procedures of collection, storage and assay are duplicated. To provide the greatest comparability of information it may be necessary to seek more methodology details than Hill provided in either this publication or in previous manuscripts (Hill and Fleming, 1982).

A few comparisons of methodologies in our laboratory demonstrated that seemingly minor differences in technique can influence the final ChE activity rates significantly. For example, homogenizer type, speed and duration of blending can influence the activity rate. A comparison of brain ChE from normal bobwhite brain prepared with either a power-driven tissue grinder (Pyrex®, Corning Glass Works, Corning, New York 14831, USA) or a VirTis® 45 power homogenizer (The VirTis Co., Inc., Gardiner, New York 12525, USA) demonstrated higher activities in the VirTis homogenates ($\bar{x}$ = 5.49 and 6.35 μmole hydrolyzed/min/g, SE = 0.17 and 0.16, n = 10; for grinder and VirTis, respectively; t = 3.77, $P < 0.005$). Glass versus plastic cuvettes also result in different activity values. In our experience, plastic cuvettes (A. H. Thomas Co., Philadelphia, Pennsylvania 19105, USA) consistently give lower Δ-absorbance/min values among readings of the same sample than do glass cuvettes (VWR Scientific, Inc., San Francisco, California 94120, USA) ($\bar{x}$ = 0.339 and 0.360, SE = 0.015 and 0.017, n = 5; for plastic and glass, respectively; t = 1.04, $0.20 < P < 0.10$). Another source of variability is preparation of the brain homogenate prior to performing the assay. Whole homogenate can be used, either mixed immediately prior to initiating the reaction or allowed to settle for 30 min. An alter-

nate method requires centrifugation of the homogenate to remove particulate matter prior to conducting the assay. Centrifugation results in decreased activity values (μmole hydrolyzed/min/g brain) compared to either settled or shaken whole homogenate ($\bar{x}$ = 2.2, 4.8, 5.0; SE = 0.75, 1.9, 1.9; n = 10; for centrifuged, uncentrifuged-settled, and uncentrifuged-shaken, respectively; t = 1.4, P = 1.0, uncentrifuged versus centrifuged). Therefore, it is apparent that differences in storage, handling and/or assay techniques from those described by Hill (1988) will introduce sources of variability that confound true insecticide-related differences that may exist between published control values and the field samples in question. Eliminating as many methodological differences as possible will increase the accuracy of comparisons between data sets.

The publication of Hill's (1988) data is especially timely as it comes when many wildlife toxicologists are considering the usefulness of a standardized ChE assay methodology. We also advocate along with Mineau and Peakall (1987) that standardized methods and quality assurance programs are needed to allow for more efficient use of data bases such as this. In addition to standardizing many of the collection, storage and assay steps already mentioned, it would be useful for laboratories to routinely conduct the assay on a ChE standard. This would verify the repeatability of the assay method from day to day or year to year. Further, the availability of ChE values derived from a commonly used standard would give laboratories the ability to demonstrate comparability between their values and those obtained by other labs or published as normal value data bases.

Correct interpretation and application of values generated by the ChE assay are of equal importance as a standardized assay technique. Data from brain assays have been used to determine if an individual died from exposure to a ChE-inhibitor (Hill and Fleming, 1982), if an animal has experienced a sublethal exposure to such a compound (Ludke et al., 1975) or if populations of animals have been negatively impacted by exposure to antiChE's (Busby et al., 1987). The use of this assay as an indicator of death due to exposure to a ChE-inhibitor is probably the most reliable application. In general, by the time death results, brain ChE values have been severely depressed. It is more difficult to determine what level of suppression is indicative of a sublethal exposure to a ChE-inhibitor. The generally accepted number has been a value depressed 20% of normal (Ludke et al., 1975). However, most workers have come to realize that exposure to the same dose of ChE-inhibitor can result in widely varying levels of depression between individuals without them exhibiting any obvious clinical signs. Human and veterinary clinicians commonly consider clinical pathology functions to be abnormal only if they exceed plus or minus two standard deviations of the mean. Hill (1988) suggests that this approach would give a more conservative diagnostic threshold.

When dealing with wildlife populations, it is as important to know if the population as a whole is depressed compared to a control population as it is to know which individuals had the lowered brain activity. We conducted a survey of brain activity of live passerines immediately following a spray event with a carbamate and found 27% (17/62) were 10 to 20% depressed compared to their before spray controls and only two birds were depressed greater than two standard deviations. However, a two-way analysis of variance taking into account age and distribution factors demonstrated that the carbamate treatment significantly reduced brain ChE activity ($P < 0.05$).

We would like to emphasize that measurement of brain ChE activity is only one of many methods that should be used to assess effects of exposure of terrestrial wildlife to organophosphate or carbamate insecticides. In the context of population studies, these data are most useful in de-

termining if an animal has been exposed to the insecticide and, if found dead, if death realistically could be attributed to the compound or was due to an unrelated event. Busby et al. (1987) in a recently published paper concluded that the use of Zectran® (mexacarbate) did not pose a threat to forest songbirds even though laboratory data showed significant ChE depression at doses similar to those used in the field. They based their conclusions on samples from singing males harvested two to three days after the spray event. These birds had brain ChE values <20% depressed compared to a small control group harvested simultaneously. However, it is possible that the sample collection was biased toward healthy males since it can be difficult to find sick or dead songbirds in the field (Grue and Shipley, 1981; Mineau and Peakall, 1987). It would have been useful to have employed other methods of assessing changes in the songbird populations and not used brain ChE measurements as the sole criterion for determining safety of application.

Another approach to using ChE activity as an indicator of sublethal exposure is sequential sampling of blood (plasma) ChE activity. Although there is a greater amount of between animal variability in plasma ChE as compared to brain ChE, repeated measures within an animal are relatively stable and provide a more sensitive measurement of acute, nonlethal effects of ChE-inhibitors (A. Fairbrother, unpubl. data). A great deal more information is needed before plasma ChE analyses can be done routinely for wild animals. It is necessary to determine which types of esterases (e.g., butyrlcholinesterase versus acetylcholinesterase) are present in the plasma of various species in order to know which substrate(s) to use in the reaction. It also is necessary to understand the differential responses of the esterases to anticholinesterase compounds so results can be interpreted in a meaningful manner.

An additional method to the use of control values for determining if the depressed brain ChE activity is due to a reversible inhibitor such as an organophosphate or carbamate is the use of the 2-PAM reactivation technique (Martin et al., 1981; Hooper et al., 1986). Briefly, this method requires measuring the ChE activity in brain alone, then incubating the homogenate with 2-PAM (pyridine-2-aldoxime methiodide) for a set amount of time and taking another reading. If the activity had been lowered by an organophosphate or carbamate, the ChE activity should increase significantly (up to control levels) following 2-PAM incubation. This might be particularly helpful in cases of ChE inhibition when death did not result as well as determining if "normal controls" taken concurrently or prior to the current investigation had been unknowingly exposed to a reversible inhibitor. Irreversible inhibitors such as mercuric chloride (Dieter, 1974) or methyl mercury (Dieter and Ludke, 1975) cause ChE depression that is unaffected by 2-PAM.

In summary, we think that Hill's (1988) efforts to collate and publish his data base of "normal" brain ChE values for so many avian species is commendable but the data must be used with care. A standardized method outlining techniques for collection and storage as well as assay parameters such as cuvette type and homogenization procedures is essential before we can utilize data bases generated by Hill and others to their full extent. The technique should be as rigorous and biologically realistic as possible yet still capable of being conducted in a field laboratory by personnel with minimal training in biochemical techniques. Most importantly, proper quality control and assurance procedures should be adhered to by all laboratories conducting the tests, regardless of whether their purpose is field diagnostics or rigorous research. Until such time as interlaboratory data can be more comparable, we will need to continue to rely on our own data sets, the 2-PAM reactivation technique, or comparisons of exposed birds to unexposed or "control" animals collected

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